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(57) Abstract

Compositions and method for inducing expression of a desired DNA sequence in a stable transformed plant expressing a hybrid transcription factor, comprising a fusion of the DNA–binding domain and a transcription activation domain, which is an effector of expression of the desired DNA sequences controlled by a synthetic promoter, said synthetic promoter preferably comprising concatemeric copies of the cis–acting site recognized by DNA–binding domain of the hybrid transcription factor, fused to a promoter.

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Method and Compositi ns Us ful for the Activation of Silent Transgenes

The invention relates to compositions for controlling expression of genetic sequences, and to a method of use thereof. More particularly the invention relates to inducing expression of an antisense nucleic acid sequence that specifically inactivates expression of a target gene.

A fundamental problem with existing technology for inactivating gene expression relies on use of constitutive promoters, which drive expression of the incoming transgene (or gene fragment) all the time. Thus, transformed cells that receive the incoming transgene are also immediately subjected to effects of its expression. In cases where the incoming transgene can inhibit expression of an essential endogenous gene, all transformed cells are killed soon after transformation. It is desirable, therefore, to have inducible control of the expression of introduced DNA sequences.

However, tight, inducible control of the expression of introduced genes has not yet been achieved in whole plants. Such control could have several uses, including practical ones such as regulating genes for controlling fertility, and more basic ones such as probing function by knock-out of a novel gene.

Many positive transcriptional regulatory factors are modular, consisting of a DNA-binding domain and an activation domain that interacts with components of the transcriptional machinery assembling at the promoter (Ptashne, 1988; Swaffield *et al.*, 1995). Fusing combinations of these elements, derived from different kingdoms, has resulted in production of diverse hybrid factors having defined DNA-binding specificities and transcription activation function for the target organism in question. For instance, in transient expression experiments in tobacco protoplasts, transcription factors derived from the yeast GAL4 transcriptional activator have been shown to activate transcription from a reporter gene controlled by a synthetic promoter consisting of multiple GAL4 DNA binding sites and a TATA element derived from a promoter recognized by plant cells (Ma *et al.*, 1988). The function of hybrid transcriptional activators and activator mutants has also been studied through high-velocity microprojectile delivery of genes into the aleurone layer of maize seed. A GAL4 DNA binding domain fused to the acidic activation domain of herpes simplex virus VP16 protein or the functionally related maize regulatory protein C1 was

shown to stimulate the expression of a GAL4-dependent reporter gene when both transactivator and reporter genes were introduced on microprojectiles (Goff *et al.*, 1991). A chimeric transcriptional activator composed of the DNA binding domain of bacteriophage 434 fused to the VP16 activation domain was shown to activate gene expression of a reporter gene driven by a synthetic promoter consisting of 434 operators fused to a minimal 35S promoter when transiently introduced into tobacco protoplasts (Wilde *et al.*, 1994). Together these studies establish that DNA binding domains from heterologous factors can bind to synthetic promoters containing appropriate binding sites on naked DNA templates introduced into plant cells, and non-plant activation domains can productively interact with the transcription machinery of the plant when covalently linked to a DNA binding domain.

Although analysis of transgenes introduced transiently into host cells can be useful to make preliminary determinations of gene function, stable transformation would be a more broadly applicable system for studying plant gene expression. It is reported, however, that the GAL4 DNA binding domain is inefficiently expressed in plants (Reichel, C. et al., (1995) "Inefficient Expression of the DNA-Binding Domain of GAL4 in Transgenic Plants" Plant Cell Reports 14(12):773-776). An ideal regulatory system for controlling transgene expression in plants would have little or no background expression in the absence of a functional transcription factor and high expression in the presence of a functional transcription factor.

What is needed, therefore, are compositions and a method useful for providing inducible expression of introduced DNA sequences in stably transformed plants.

The present invention answers a long felt yet unfulfilled need in the art by providing a method, compositions and transgenic plants containing heterologous DNA sequences for stably introducing into a plant hybrid transcription factors and a synthetic promoter to induce expression of an activatable DNA sequence, such as a transgene. Importantly, the invention described herein effectively separates insertion of the transgene from its activity, thus permitting recovery of otherwise lethal transformants, followed later by their activation. A utility of the invention is for investigating gene function, for example by identifying genes that are important for plant growth, development and viability. A particularly important feature of the invention is the ability to induce the expression of antisense DNA sequences, or dominant inhibitors, such as a translatable or untranslatable sense sequence capable of disrupting gene function, in stably transformed plants to positively identify one or more genes essential for normal growth and development of a plant.

The invention encompasses hybrid transcription factor genes, synthetic promoters, activatable DNA sequences and cells, tissues and plants containing such genes, promoters and DNA sequences, and method of use thereof to determine plant gene function. A hybrid transcription factor gene and a synthetic promoter are selected or designed such that the DNA binding domain of the hybrid transcription factor is capable of binding specifically to the synthetic promoter to activate expression of an activatable DNA sequence driven by the synthetic promoter.

More specifically, the invention encompasses a first cell or tissue, preferably a plant cell or plant tissue, or a plant, containing a sequence encoding a hybrid transcription factor necessary to activating a synthetic promoter, and a second cell or tissue, preferably a plant cell or plant tissue, or a plant, containing the synthetic promoter, wherein the first and the second cell, tissue or plant can be manipulated to create a cell, tissue or plant containing the both the sequence encoding the hybrid transcription factor and the synthetic promoter. Desirably, the second cell, tissue or plant contains the synthetic promoter operably linked to an activatable DNA sequence, whose expression is driven by the synthetic promoter when the promoter is activated by the hybrid transcription factor. In another embodiment the hybrid transcription factor is designed or selected so as to be controllable by an independent factor, such as, for example, estrogen.

The method of the invention encompasses combining the first and the second cells, tissues or plants to form a stable transgenic plant containing both the hybrid transcription factor gene and the synthetic promoter such that the activatable DNA sequence is expressed in the plant. One embodiment permits one skilled in the art to activate expression of an otherwise silent gene (or gene fragment) of interest, such as an antisense gene, by sexually introducing a gene encoding a transcriptional factor that specifically activates expression from a synthetic promoter controlling the gene of interest. When the gene of interest is capable of inactivating expression of an endogenous gene, progeny of the sexual cross between the plant containing the gene of interest and the effector plant will be unable to normally express the endogenous gene. Plant genes essential for normal growth or development can be identified in this manner. The identification of such genes provide useful targets for screening compound libraries for effective herbicides.

The invention described herein demonstrates that a hybrid transcription factor can function effectively to control gene expression in stably transformed plants. The following detailed description is the first evidence that hybrid transcription factors can effectively

activate gene function in whole plants, thereby providing a useful system for positively identifying genes essential for plant growth.

The present invention provides an important new method for investigating gene function in plants. The invention is particularly useful for identifying endogenous plant DNA sequences that are necessary or important for growth, development or viability. The identification of such DNA sequences provides information essential for the rational and efficient development of safe, effective, economically feasible and environmentally sound products to solve important agricultural problems. In one embodiment the invention can be used to test for function of endogenous genes by knocking out their expression. In particular, it can be used to verify potential herbicide target genes, by ascertaining whether a gene of interest is essential for normal growth and development of the plant. This provides an early and essential link in the screening and development chain and provides the motivation to marshal resources and direct the expenditure of effort to identify and test the most potent compounds, thereby providing an immediate benefit to the public.

In order to provide a full, clear, concise and exact description of the claimed invention, definitions for the following terms as they are used herein are provided.

Activatable DNA construct - refers to a DNA sequence, or a recombinant construct containing the DNA sequence and a synthetic promoter, which when introduced into a cell, desirably a plant cell, is not expressed, i.e. it is silent, unless a complete hybrid transcription factor capable of binding to and activating the synthetic promoter, is present. The activatable DNA construct subsequently is introduced into cells, tissues or plants to form stable transgenic lines capable of expressing the activatable DNA sequence, as described more fully below.

Activatable DNA sequence - refers to a DNA sequence that regulates the expression of genes in a genome, desirably the genome of a plant. The activatable DNA sequence is complementary to the target DNA sequence endogenous in the genome. When the activatable DNA sequence is introduced and expressed in a cell, it inhibits expression of the target DNA. An activatable DNA sequence useful in the present invention includes those encoding or acting as dominant inhibitors, such as a translatable or untranslatable sense sequence capable of disrupting gene function in stably transformed plants to positively identify one or more genes essential for normal growth and development of a plant. A preferred activatable DNA sequence is an antisense DNA sequence. The mechanism by

which antisense sequences work is not known, presumably the antisense RNA binds to target gene RNA to inhibit the expression of the target DNA gene product. It is possible that such RNA:RNA complexes inhibit the binding or function of translational machinery, alternatively it is possible that such RNA:RNA complexes are rapidly degraded. Other mechanisms are possible. Desirably the target gene encodes a protein, such as a biosynthetic enzyme, receptor, signal transduction protein, structural gene product or transport protein that is essential to the growth or survival of the plant. The interaction of the antisense RNA sequence and the target gene RNA results in substantial inhibition of the expression of the target DNA sequence so as to kill the plant, or inhibit normal plant growth or development.

Expression refers to the transcription and/or translation of an endogenous gene or a transgene in plants. In the case of antisense constructs, for example, expression may refer to the transcription of the antisense DNA only.

Gene refers to a coding sequence and associated regulatory sequences wherein the coding sequence is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Examples of regulatory sequences are promoter sequences, 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

Gene of interest refers to any gene which, when transferred to a plant, confers upon the plant a desired characteristic such as antibiotic resistance, virus resistance, insect resistance, disease resistance, or resistance to other pests, herbicide tolerance, improved nutritional value, improved performance in an industrial process or altered reproductive capability. The "gene of interest" may also be one that is transferred to plants for the production of commercially valuable enzymes or metabolites in the plant.

Heterologous DNA sequence - refers to a DNA sequence not naturally associated with the host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring DNA sequence.

Marker gene refers to a gene encoding a selectable or screenable trait

Operably linked to/associated with refers to a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence.

Minimal promoter - refers to promoter elements, particularly a TATA element, that are inactive, or which have greatly reduced promoter activity in the absence of upstream activation. In the presence of suitable transcription factor the minimal promoter functions to permit transcription.

Plant refers to structural and physiological unit of the plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, or a plant organ.

Plant cell refers to any plant, particularly to seed plants

Plant material refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, pollen tubes, ovules, embryo sacs, egg cells, zygotes, embryos, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant

Promoter a DNA sequence that initiates transcription of an associated DNA sequence. The promoter region may also include elements that act as regulators of gene expression such as activators, enhancers, and/or repressors

Recombinant DNA: molecule a combination of DNA sequences that are joined together using recombinant DNA technology

Recombinant DNA technology refers to procedures used to join together DNA sequences as described, for example, in Sambrook et al., 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Selectable marker gene refers to a gene whose expression in a plant cell gives the cell a selective advantage. The selective advantage possessed by the cells transformed with the selectable marker gene may be due to their ability to grow in the presence of a negative selective agent, such as an antibiotic or a herbicide, compared to the growth of non-transformed cells. The selective advantage possessed by the transformed cells, compared to non-transformed cells, may also be due to their enhanced or novel capacity to utilize an added compound as a nutrient, growth factor or energy source. Selectable marker gene also refers to a gene or a combination of genes whose expression in a plant cell gives the cell both, a negative and a positive selective advantage.

Stably transformed - refers to a cell, desirably a plant cell, containing at least one heterologous DNA sequence, wherein the heterologous DNA sequence is maintained in the cell, and is functional under the appropriate conditions for its intended use, and is heritable to subsequent generations. The term specifically includes those cells into which the heterologous DNA sequence is initially introduced, by whatever means, and to

subsequently derived cells, tissues, plants and seeds containing the heterologous DNA sequence. The latter are also referred to as stable transgenic lines.

synthetic refers to a nucleotide sequence comprising structural characters that are not present in the natural sequence. For example, an artificial sequence that resembles more closely the G+C content and the normal codon distribution of dicot and/or monocot genes is said to be synthetic.

Transformation (r) refers to introduction of a nucleic acid into a cell. In particular, the stable integration of a DNA molecule into the genome of an organism of interest.

As described more fully below, the invention encompasses hybrid transcription factor genes, synthetic promoters, activatable DNA sequences and cells, tissues and plants containing such genes, promoters and DNA sequences, and methods of use thereof. The hybrid transcription factor gene and the synthetic promoter are selected or designed such that the DNA binding domain of the hybrid transcription factor is capable of binding specifically to the synthetic promoter to turn on expression of an activatable DNA sequence driven by the synthetic promoter.

In one embodiment the invention is based on sexual crossing to achieve inducible gene activation, relying on an transcriptional effector line to activate expression of a silent transgene under the control of a synthetic, activatable promoter. Traits whose expression can be controlled with this system include both non-plant genes, translated or untranslated sense genes for transcriptional control elements such as endogenous promoters, or post-transcriptional control elements, and antisense genes to knock out expression of endogenous genes (*e.g.* AdSS, which is described below). In one case the transcriptional effector line contains a DNA sequence encoding a hybrid transcription factor, as described more fully below, which is crossed with a cell, tissue or plant line containing the synthetic promoter operably linked to the activatable DNA sequence. In another case, the transcriptional effector line contains a DNA sequence encoding one portion of a hybrid transcription factor, wherein the protein encoded thereby is capable of forming a hybrid transcription factor through peptide-peptide interaction with the conjugate part of the hybrid transcription factor. The synthetic promoter, activatable DNA sequence and the DNA sequence encoding the corresponding conjugate part of the hybrid transcription factor are contained in a separate line. In yet another case, the hybrid transcription factor is rendered ineffective by an inhibitor, thereby preventing it from activating the synthetic promoter, for

example by blocking functional binding of the hybrid transcription factor to the synthetic promoter. Consequently, the activatable DNA sequence is not expressed unless and until the hybrid transcription factor inhibitor is removed.

Based on this disclosure, one skilled in the art will appreciate that any DNA sequence or gene of interest can be controlled in this way.

This system is especially useful for allowing expression of traits that might otherwise be unrecoverable as constitutively driven transgenes. For instance, foreign genes with potentially lethal effect, or antisense genes or dominant negative mutations designed to abolish function of essential genes, while of great interest in basic studies of plant biology, present inherent experimental problems. Decreased transformation frequencies are often cited as evidence of lethality associated with a particular constitutively driven transgene, but negative results of this type are laden with alternative trivial explanations. A system of the type described here allows stable maintenance and propagation of a test transgene separate from its expression. This ability to separate transgene insertion from expression is crucial for firm conclusions about essentiality of gene function to be drawn. The present invention , therefore, is a substantial contribution to the art.

Variation in severity of phenotype can be achieved by examining the phenotypes of multiple independent activatable lines crossed to a single activator. By relying on position effect to provide varying levels of expressibility from the different transgenic loci, it is possible to obtain a phenocopy of an allelic series for a specific trait. This is illustrated below in Example 2, which shows that a diversity of expression levels from an antisense gene designed to knock out an essential metabolic function results in plant lines with varying severity of phenotype. Similarly, other traits will also be inactivatable to varying degrees in independent lines.

Optionally, expression of particular traits is achieved by further controlling the expression of the hybrid activator gene with appropriate promoters, for example promoters regulated in developmental time or space. Depending on the stringency of control of the promoter in question, assessing the function of a gene of interest in specific cell types, tissues, or organs or at specific times in development is possible. For instance, the requirement for function of a gene in embryo development could be tested by activating an antisense transgene with a factor driven by a promoter known to be expressed strongly in developing seeds. Such an approach has been widely used in *Drosophila*, usually by inserting the a GAL4 effector construct at random to obtain fusions to various genomic enhancers directing expression in different cell and tissue types (Brand and Perrimon,

1993). Similarly, the DNA constructs of the present invention provide additional control of gene expression by way of chemically inducible promoters, for example using steroid-inducible gene expression as exemplified by Schena, et al. (Proc. Natl. Acad. Sci., USA, Vol. 88, pp 10421-10425, December 1991).

Further levels of modulation of expression are achieved by choosing an activation domain of appropriate strength for a specific application. Recently, plant transcriptional activation domains of net positive or negative charge were identified in a yeast functional screen (Estruch *et al.*, 1994). Fusion of these domains to the DNA binding domain of GAL4 yielded proteins capable of activating a GAL4-dependent promoter gene when both were introduced into maize or tobacco cells on microprojectiles. Thus, a variety of different activation domains are identifiable by direct functional or structural screens.

Although the work described here is exemplified in *Arabidopsis*, the transactivation *per se* is not limited to this one species. Using appropriate promoters, the system described here functions in any species, including commercially important plants, including but not limited to corn, rice, wheat, sugar beet, barley, rye, cotton, rape, oats, sorghum, millet, turf grasses and ornamentals.

Hybrid Transcription Factor Gene

A hybrid transcription factor gene comprises DNA sequences encoding 1) a DNA-binding domain and 2) an activation domain that interacts with components of the transcriptional machinery assembling at the promoter. Gene fragments are joined, typically such that the DNA binding domain is toward the 5' terminus and the activator domain is toward the 3' terminus, to form a hybrid gene whose expression produces a hybrid transcription factor. One skilled in the art is capable of routinely combining various DNA sequences encoding DNA-binding domains with various DNA sequences encoding activation domains to produce a wide array of hybrid transcription factor genes.

Examples of DNA sequences that can be used to make hybrid transcription factors useful in the invention include, but are not limited to those encoding the DNA binding domain of GAL4, bacteriophage 434, *lexA*, *lacI*, and phage lambda repressor.

Examples of DNA sequences that can be used to make hybrid transcription factors useful in the invention include, but are not limited to those encoding the acidic activation domain of herpes simplex VP16, maize C1, and P1. In addition, suitable activation domains can be isolated by fusing DNA pieces from an organism of choice to a suitable DNA binding domain and selecting directly for function. (Estruch et al., 1994, incorporated by reference in

its entirety). Domains of transcriptional activator proteins can be swapped between proteins of diverse origin. (Brent and Ptashne (1985) Cell 43:729-736).

A desirable hybrid transcription factor gene comprises DNA sequences encoding the GAL4 DNA binding domain fused to the maize C1 activation domain. One skilled in the art can use routine molecular biology and recombinant DNA technology to make desirable hybrid transcription factor genes.

Synthetic Promoter

A synthetic promoter comprises at least one DNA binding site recognized by the DNA binding domain of the hybrid transcription factor, and a minimal promoter, preferably a TATA element derived from a promoter recognized by plant cells. More particularly the TATA element is derived from a promoter recognized by the plant cell type into which the synthetic promoter will be incorporated. Desirably, the DNA binding site is repeated multiple times in the synthetic promoter so that the minimal promoter may be more effectively activated, such that the activatable DNA sequence associated with the synthetic promoter is more effectively expressed.

Examples of DNA binding sites that can be used to make synthetic promoters useful in the invention include but are not limited to the upstream activating sequence (UAS_G) recognized by the GAL4 DNA binding protein, the *lac* operator, and the *lexA* binding site. Examples of promoter TATA elements recognized by plant cells include those derived from CaMV 35S, the maize *Bz1* promoter, the UBQ3 promoter, Agrobacterium nopaline synthase or mannopine synthase promotor such as SuperMAS, maize Bz1 promotor, UBQ3 promotor, hsp80 from Brassica oleracea and arabidopsis actine-2 promotor.

A desirable synthetic promoter comprises a truncated CaMV 35S sequence containing the TATA element (nucleotides -59 to +48 relative to the start of transcription), fused at its 5' end to approximately 10 concatemeric direct repeats of the upstream activating sequence (UAS_G) recognized by the GAL4 DNA binding domain. One skilled in the art can use routine molecular biology and recombinant DNA technology to make desirable synthetic promoters.

Activatable DNA sequence

An activatable DNA sequence encompasses any DNA sequence for which stable introduction and expression in a plant cell is desired. Particularly desirable activatable DNA sequences are sense or antisense sequences, who expression results in decreased

expression of their endogenous counterpart genes, thereby inhibiting normal plant growth or development.

The activatable DNA sequence is operably linked with the synthetic promoter to form the activatable DNA construct. The activatable DNA sequence in the activatable DNA construct is not expressed, i.e. it is silent, in transgenic lines, unless a hybrid transcription factor capable of binding to and activating the synthetic promoter, is also present. The activatable DNA construct subsequently is introduced into cells, tissues or plants to form stable transgenic lines expressing the activatable DNA sequence, as described more fully below.

Cell, Tissue or Plant Containing the Hybrid Transcription Factor, or the Synthetic Promoter and Activatable DNA sequence

The invention also encompasses a first and a second cell or tissue, preferably a plant cell or plant tissue, or plant, containing a hybrid transcription factor gene and an activatable DNA construct, respectively. The first cell, tissue or plant and the second cell, tissue or plant are selected such that they can be manipulated to create a plant cell, plant tissue or plant containing the hybrid transcription factor gene and expressing the hybrid transcription factor, and also containing and activating the synthetic promoter of the activatable DNA construct so as to express the activatable DNA sequence driven by the promoter.

Hybrid transcription factor genes and activatable DNA constructs above are introduced into a cell, tissue or plant by methods well known and routinely used in the art, including but not limited to crossing, *Agrobacterium*-mediated transformation, Ti plasmid vectors, direct DNA uptake such as microprojectile bombardment, liposome mediated uptake, micro-injection and the like.

Transgenic plant lines containing the hybrid transcription factor gene are created using, for example, *Agrobacterium*-mediated transformation. Primary transformants (T1 generation) are screened for the ability to activate expression from a corresponding synthetic promoter (i.e. a synthetic promoter that is activatable by the hybrid transcription factor) by transiently transforming them with a reporter construct. Routine RNA gel blot analysis is used to confirm that the transformants express the hybrid transcription factor gene. The transgenic nature of the lines is further tested in the T2 generation for segregation of kanamycin resistance (or other appropriate selectable marker gene carried on the inserted DNA) as a single locus after selfing. The presence of a single T-DNA insert is confirmed by genomic DNA gel blot analysis in lines that show 3:1 segregation. These

lines may be further analyzed for expression of the hybrid transcription factor gene by RNA gel blot analysis. Several T2 plants are selfed to obtain T3 progeny, which are screened for homozygosity of the inserted hybrid transcription factor gene.

Transgenic lines containing a synthetic promoter activatable by a desired hybrid transcription factor are prepared in a similar fashion by *Agrobacterium*-mediated transformation. The transgenic lines containing the synthetic promoter and the activatable DNA sequence (the activatable DNA construct), and optionally a selectable marker are prepared by standard methods. Optionally, the transgenic lines are selected for the marker to confirm the presence of the activatable DNA construct.

F1 Plants Containing the Hybrid Transcription Factor, and the Activatable DNA Construct

F1 plants containing both the hybrid transactivator gene and the activatable DNA construct are generated by cross-pollination and selected for the presence of an appropriate marker, such as kanamycin. In contrast to plants containing the activatable DNA construct alone, the F1 plants generate high levels of activatable DNA sequence expression product, comparable to those obtained with strong constitutive promoters such as CaMV 35S.

A useful assay method of the invention comprises

a) crossing a first stably transformed plant comprising a hybrid transcription factor gene encoding a hybrid transcription factor capable of activating a synthetic promoter, when said synthetic promoter is present in the plant, and wherein the plant is homozygous for the hybrid transcription factor;

b) with a second stably transformed plant comprising an activatable DNA sequence and a synthetic promoter that is activatable by the hybrid transcription factor, wherein the DNA sequence is expressed in the presence of the hybrid transcription factor to yield F1 plants expressing the DNA sequence; and

c) determining the effect of expression of the DNA sequence on the F1 plants.

Preferred is said assay wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from a C1 gene of maize.

Further preferred is said assay, wherein the minimal promoter is selected from the group consisting of the CaMV 35S minimal promoter, the maize *Bz1* promoter and the UBQ3 promoter.

Preferred is said assay, wherein the synthetic promoter sequence comprises a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

Preferred is said assay, wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from the C1 gene of maize, and wherein the activatable DNA construct comprises a synthetic promoter sequence comprising a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

Another useful method of the invention provides for identifying essential plant gene inhibitors, such as the plant AdSS gene comprising,

a) reacting a plant AdSS enzyme and AdSS substrate in the presence of a suspected inhibitor of AdSS enzymatic function; and

b) comparing the rate of AdSS enzymatic reaction in the presence of the suspected inhibitor to the rate of AdSS enzymatic reaction under the same conditions in the absence of the suspected inhibitor, to determine whether the suspected inhibitor inhibits AdSS.

A preferred embodiment of the invention encompasses a plant comprising a hybrid transcription factor gene and an activatable DNA construct, wherein the hybrid transcription factor encoded by the hybrid transcription factor gene is capable of activating the synthetic promoter of the activatable DNA construct to induce expression of an operably linked antisense DNA sequence, wherein the plant is stably transformed with the hybrid transcription factor and with the activatable DNA construct.

Preferred is said plant, wherein the hybrid transcription factor gene comprises

a DNA binding domain derived from a gene selected from the group consisting of a GAL4 gene of yeast, bacteriophage 434, *lexA*, *lacI* and lambda phage repressor;

a transcription activation domain derived from a gene selected from the group consisting of herpes simplex VP16, maize C1 and P1;

the activatable DNA construct comprises a minimal promoter selected from the group consisting of the CaMV 35S minimal promoter, the maize *Bz1* promoter and the UBQ3 promoter.

Further preferred is said plant, wherein the synthetic promoter sequence comprises a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

Further preferred is said plant, wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from the C1 gene of maize, and wherein the activatable DNA construct comprises a synthetic promoter sequence comprising a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

Further preferred is said plant, wherein the activatable DNA sequence is an AdSS antisense sequence.

EXAMPLES

The examples disclosed below demonstrate that a hybrid transcription factor can function effectively to control gene expression in stably transformed plants. The invention is further described and will be further understood by one skilled in the art in light of the following non-limiting examples.

Example 1 - Expression of a Silent Reporter Gene in Stable Transgenic Plants

This example illustrates that crossing a transgenic plant line expressing a GAL4/C1 hybrid factor to a line containing a reporter transgene controlled by an appropriate synthetic promoter results in strong induction of reporter gene expression. The appropriate genes for testing the system in *Arabidopsis* were constructed as described more fully below.

A hybrid transcription factor gene is constructed from components of the *GAL4* and *C1* genes previously shown to contain the DNA-binding and transcription activation functions, respectively. (The construct used for plant transformation pAT 53 contains a left border sequence coupled to a 35S promotor operably linked to a hybrid transcription faction comprised of a GAL4 DNA binding domain coupled to a C1 activation domain with a 35S 3' terminator sequence and a pNOS/NPT/nos 3' selectable marker cassette bounded by a right border sequence). The N-terminal 147 amino acids of the encoded protein derive from *GAL4*, and the C-terminal 101 amino acids are derived from the carboxy-terminal amino acids 173-273 of *C1*. A similar combination had previously been shown to function in transient assays (Goff et al., 1991).

A synthetic promoter designed to be activatable by this factor is constructed using Bz1 TATA element (optionally the truncated CaMV 35S promoter, containing the TATA element (nucleotides -59 to +48 relative to the start of transcription) is used), fused at its 5' end to 10 concatemeric copies of the upstream activating sequence (UAS_G) recognized by GAL4 protein. (The construct pAT 73 contains a left border sequence coupled to a 35S promotor operably linked a dihydrofolate reductase coding sequence linked to a 35S 3' terminator, which in turn is ligated to a 10-fold concatenated GAL4 binding site construct containing a TATA element operably linked to a GUS reporter element with a 35S 3' terminator, all of which is bounded by a right border sequence.) To evaluate the efficacy of the system in stable transformants, a reporter gene is selected as the activatable DNA sequence, for example a modified *E. coli uidA* (β-glucuronidase; GUS) coding sequence

driven by the synthetic UAS_G/TATA promoter. The GUS gene is considered a model reporter gene for expression in that its gene product is readily detected and quantified.

Transgenic *Arabidopsis* plant lines containing the hybrid transcription factor gene were created using *Agrobacterium*-mediated transformation. Primary transformants (T1 generation) were screened for ability to activate expression from the synthetic UAS_G/TATA promoter by transiently transforming them with a luciferase reporter construct. Approximately half of the T1 transformants tested showed luciferase activity after microprojectile bombardment. RNA gel blot analysis confirmed that these transformants expressed the GAL4/C1 gene. These lines were further tested in the T2 generation for segregation of kanamycin resistance (the selectable marker gene carried on the T-DNA) as a single locus after selfing. Presence of a single T-DNA insert is confirmed by genomic DNA gel blot analysis in lines that showed 3:1 segregation (data not shown). These lines are further analyzed for expression of the *GAL4/C1* gene by RNA gel blot analysis. RNA blot analysis shows that both lines expressed detectable levels of stable RNA derived from the transgene. A single effector line, designated pAT53-103, was chosen for further experiments, and several T2 plants were selfed to obtain T3 transgenic progeny which were screened for homozygosity of the T-DNA insert.

Transgenic *Arabidopsis* plant lines containing the UAS_G/TATA/GUS gene were created using Agrobacterium-mediated transformation, and were selected on methotrexate and screened for homozygosity. Two lines, designated pAT73-309 and -346, were analyzed for GUS activity, and found to have very low amounts, not significantly different from assay background (Table 1). F1 plants containing both the hybrid transactivator gene and the activatable reporter gene were generated by cross-pollination and selected on kanamycin. In contrast to plants containing the reporter gene alone, the F1 plants produced very high levels of GUS activity, comparable to those obtained with strong promoters such as CaMV 35S (Table 1).

Table 1. β-glucuronidase activity in F1 plants.

Transformant		GUS activity * (nmol MU/min/mg protein)
35S/GUS	line 7	17.6 ± 4.3
	line 105	19.1 ± 7.2
	line 115	12.1 ± 3.7
untransformed Nossen		0.03 ± 0.01
pAT53-103		0.01 ± 0.0
pAT73-309		0.01 ± 0.01
pAT73-309 x pAT53-103 F1		4.97 ± 3.41
pAT73-346		0.01 ± 0.0
pAT73-346 x pAT53-103 F1		6.02 ± 2.3

* GUS activity was measured for 20 plants of each line or F1 cross (mean ± standard deviation).

Example 2 - Expression of Silent Antisense DNA Sequences in Stable Transgenic Plants

Additionally, the invention can be used to investigate gene function, by inducing expression of an antisense gene that specifically inactivates expression of a test gene. The invention is used to drive expression of an antisense gene to eliminate gene function. The gene encoding adenylosuccinate synthetase (AdSS), one of two steps in *de novo* purine biosynthesis that converts IMP to AMP, was used as an activatable DNA sequence. AdSS has recently been implicated as the target of the potently herbicidal natural product hydantocidin (Cseke *et al.*, 1996; Fonné-Pfister *et al.*, 1996; Siehl *et al.*, 1996). AdSS activity is measured by standard enzymatic assays well known in the art, for example by reacting AdSS enzyme and an AdSS substrate that the AdSS enzyme is capable of catalyzing to a product measurably distinct from the substrate, and measuring the rate of

catalytic conversion of substrate to product. The conversion may be measured directly by determining the amount of substrate or product, or both present in the reaction at various times, or indirectly by measuring a label, such as radioactivity, or a color indicator associated with the substrate or the product only. Southern blot analysis reveals that the full-length cDNA used for antisense gene construction here represents a single gene in the *Arabidopsis* genome.

Based on the successful antisense DNA sequence expression achieved in the stable transgenic plants of Example 1, it was postulated that successful expression in stable transgenic plants containing both a hybrid transcription factor gene and a synthetic promoter driving expression of an AdSS antisense sequence would result in inactivation of endogenous AdSS gene expression. It was also postulated that if the AdSS antisense sequence were successfully expressed, then plant lethality would result based upon the analogy to the herbicidal effect of hydantocidin. However, prior to running such experiments, it was not clear to one skilled in the art whether expression of the antisense in stably transformed plants would occur and would result in inactivation of the AdSS gene. As described more fully below, the AdSS antisense sequence was successfully expressed in stable transgenic plants, and expression of endogenous AdSS enzyme was inhibited.

Fifteen transgenic plants containing the UAS_G/TATA/antisense AdSS construct (. were generated by *Agrobacterium* transformation. (Construct pJG261AntiAdSS contains a left border sequence coupled to a pNos/BAR/gene 7 3' selectable marker cassette linked to a 10-fold concatenated GAL4 binding site construct containing a TATA element, which is linked to AdSS antisense coding sequence terminated by 35S3' and a right border sequence.)Flowers borne on the primary transformants were crossed to pollen from the homozygous transactivator line pAT53-103. F1 seed were plated on kanamycin to select for the outcrossed progeny. These primary transformants are hemizygous for the introduced T-DNA (containing the antisense gene), which in most cases will segregate as a single Mendelian trait. Thus, the antisense gene should segregate 1:1 against a background that always contains the transactivator in the hemizygous state (except in rare contaminants from selfing, which are selected by germination on a selectable marker, such as kanamycin). In six lines, approximately 50% of the seedlings were severely retarded in growth, in some cases failing to germinate completely. Five other lines survived through true leaf expansion, but showed various growth anomalies after transfer to soil. A final four lines showed little or no abnormal phenotype.

To confirm that the severe growth retardation and lethality seen was due to presence of the antisense transgene, polymerase chain reactions were carried out using primers designed to amplify the region between the 5' end of the AdSS cDNA and the minimal 35S promoter. Gel electrophoresis demonstrates that there is a one-to-one correspondence was observed between abnormal seedlings and the antisense gene. To examine the variation in phenotype among different antisense lines, we carried out RNA gel blot hybridizations on F1 plants derived from different antisense lines. Gel blot probed with AdSS probe containing RNA from untransformed Col-0 plants , pAT53-103 plants and F1 plants derived from crossing pAT53-103X antisense AdSS shows that little AdSS RNA was detected in a line with a severe phenotype. (The most severe seedling lethal lines had to be omitted from the analysis because so little tissue was available for RNA extraction.)

The Examples will be further understood in view of the following technical descriptions.

Recombinant plasmids

pSGZL1 was constructed by ligating the GAL4-C1 *Eco*RI fragment from pGALC1 (Goff et al., 1991) into the *Eco*RI site of pIC20H. The GAL4-C1 fragment of pSGZL1 was excised with *Bam*HI-*Bgl*II and inserted into the *Bam*HI site of pCIB770 (Rothstein *et al.*, 1987) yielding pAT53.

10 UAS_G sites and the minimal 35S promoter (-59 to +1) were excised from pGALLuc2 (Goff et al., 1991) as an *Eco*RI-*Pst*I fragment and inserted into the respective sites of pBluescript, yielding pAT52. pAT66 was constructed with a three-way ligation between the *Hin*dIII-*Pst*I fragment of pAT52, a *Pst*I-*Eco*RI fragment of pCIB1716 (containing a 35S untranslated leader, GUS gene, 35S terminator) and *Hin*dIII-*Eco*RI cut pUC18. The 35S leader of pAT66 was excised with *Pst*I-*Nco*I and replaced with a PCR-generated 35S leader extending from +1 to +48 to yield pAT71.

pCIB921 contains a dihydrofolate reductase (dhfr) plant selectable marker gene inserted in the BamHI site of pCIB710 (Rothstein et al., 1987). The 35S promoter/ dhfr gene cassette of pCIB921 was excised with XbaI-EcoRI and inserted into the respective sites of pCIB730 (Rothstein et al., 1987) to make pAT58. pAT73 was constructed by inserting the EcoRI fragment from pAT71 containing 10 UASg sites/ minimal 35S promoter/ GUS/ 35S terminator into the EcoRI site of pAT58.

Plasmid pBS SK+ (Stratagene, LaJolla, CA) was linearized with *Sac*I, treated with mung bean nuclease to remove the *Sac*I site, and re-ligated with T4 ligase to make

pJG201. The UAS_G/CaMV 35S minimal promoter/GUS gene/CaMV terminator cassette was removed from pAT71 with *Kpn*I and cloned into the *Kpn*I site of pJG201 to make pJG304. pJG304 was partially digested with restriction endonuclease *Asp*718 to isolate a full-length linear fragment. This fragment was ligated with a molar excess of the oligonucleotide 5' GTA CCT CGA GTC TAG ACT CGA G 3'. Restriction analysis was used to identify a clone with this linker inserted 5' to the site, and this plasmid was designated pJG304ÆXhoI.

A fragment of the AdSS synthase cDNA clone described previously (Fonné-Pfister et al., 1996) (GenBank accession #U49389) was PCR-amplified with the oligonucleotide primers 5' GATTCGAGCTCATGTCTCTCTCTTCCCTC 3' and 5' GATTCCCATGGTGGACCTGAACCAACTC 3'. The vector pJG304ΔXhoI was digested with *Sac*I and *Nco*I to excise the GUS gene coding sequence. The AdSS PCR fragment was digested with *Sac*I and *Nco*I and ligated into pJG304ΔXhoI to make pJG304AntiAdSS.

Vector pGPTV (Becker *et al.*, 1992) was digested with *Eco*RI and *Hin*dIII to remove the nopaline synthase promoter/GUS cassette. Concurrently, the superlinker was excised from pSE380 (Invitrogen, San Diego, CA) with EcoRI and HindIII and cloned into the *Eco*R1/*Hin*dIII linearized pGPTV, to make pJG261.

pJG304AntiAdSS was cut with *Xho*I to excise the cassette containing the UAS_G/35S minimal promoter/antisense AdSS/CaMV terminator fusion. This cassette was ligated into *Xho*I-digested pJG261, such that transcription was divergent from that of the *bar* selectable marker, producing pJG261AntiAdSS.

Transgenic plants

pJG261AntiAdSS was electro-transformed into *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz and Schell, 1986), and *Arabidopsis* plants (ecotype Columbia) were transformed by infiltration (Bechtold *et al.*, 1993) using the resulting strain. Seeds from the infiltrated plants were selected on agar germination medium (Murashige-Skoog salts at 4.3 g/liter, MES at 0.5 g/liter, 1% sucrose, thiamine at 10 µg/liter, pyridoxine at 5 µg/liter, nicotinic acid at 5 µg/liter, myo-inositol at 1 mg/liter, pH 5.8) containing glufosinate (Basta®; AgrEvo) at 15 mg/liter.

Arabidopsis root explants (ecotype Nossen) were transformed with pAT53 as described (Valvekens *et al.*, 1988).

Fifteen transgenic plants containing the UAS_G/minimal CaMV 35S promoter/antisense AdSS construct were transplanted to soil and grown to maturity in the greenhouse. Flowers borne on the primary transformants were crossed to pollen from the homozygous GAL4/C1

transactivator line pAT53-103. F1 seeds were plated on germination medium containing 50 mg/liter kanamycin.

Nucleic Acid Analysis

RNA was isolated by phenol/chloroform extraction followed by LiCl precipitation as described (Lagrimini *et al.*, 1987). RNA gel blots were performed as described (Ward *et al.*, 1991). Hybridization probes were labeled with a^{32}P-dCTP by the random priming method using a PrimeTime kit (International Biotechnologies, Inc., New Haven, CT). Hybridization conditions were 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$ pH 7.0, 1 mM EDTA, 1% bovine albumin at 65C. After hybridization overnight, the filters were washed with 1% SDS, 50mM $NaPO_4$, 1mM EDTA at 65C (Church and Gilbert, 1984).

What is claimed is

1. An assay method comprising

   a) crossing a first stably transformed plant comprising a hybrid transcription factor gene encoding a hybrid transcription factor capable of activating a synthetic promoter, when said synthetic promoter is present in the plant, and wherein the plant is homozygous for the hybrid transcription factor;

   b) with a second stably transformed plant comprising an activatable DNA sequence and a synthetic promoter that is activatable by the hybrid transcription factor, wherein the activatable DNA sequence is expressed in the presence of the hybrid transcription factor to yield F1 plants expressing the activatable DNA sequence; and

   c) determining the effect of expression of the activatable DNA sequence on the F1 plants.

2. The assay of claim 1, wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from a C1 gene of maize.

3. The assay of claim 1, wherein the minimal promoter is selected from the group consisting of the CaMV 35S minimal promoter, the maize *Bz1* promoter and the UBQ3 promoter.

4. The assay of claim 1, wherein the synthetic promoter sequence comprises a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

5. The assay of claim 1, wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from the C1 gene of maize, and wherein the activatable DNA construct comprises a synthetic promoter sequence comprising a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric

copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

6. A method for identifying AdSS herbicidal inhibitors comprising,

a) reacting a plant AdSS enzyme and AdSS substrate in the presence of a suspected herbicidal inhibitor of plant AdSS enzymatic function; and

b) comparing the rate of the plant AdSS enzymatic reaction in the presence of the suspected herbicidal inhibitor, to the rate of the plant AdSS enzymatic reaction under the same conditions in the absence of the suspected herbicidal inhibitor, to determine whether the suspected herbicidal inhibitor inhibits the plant AdSS.

7. A plant comprising a hybrid transcription factor gene and an activatable DNA construct, wherein the hybrid transcription factor encoded by the hybrid transcription factor gene is capable of activating the synthetic promoter of the activatable DNA construct to induce expression of an operably linked antisense DNA sequence, wherein the plant is stably transformed with the hybrid transcription factor and with the activatable DNA construct.

8. The plant of claim 7, wherein the hybrid transcription factor gene comprises

a) a DNA binding domain derived from a gene selected from the group consisting of a GAL4 gene of yeast, bacteriophage 434, *lexA*, *lacI* and lambda phage repressor;

b) a transcription activation domain derived from a gene selected from the group consisting of herpes simplex VP16, maize C1 and P1;

c) the activatable DNA construct comprises a minimal promoter selected from the group consisting of the CaMV 35S minimal promoter, the maize *Bz1* promoter and the UBQ3 promoter.

9. The plant of claim 7, wherein the synthetic promoter sequence comprises a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10

concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

10. The plant of claim 7, wherein the hybrid transcription factor gene encodes a DNA binding domain derived from a GAL4 gene of yeast and the transcription activation domain derived from the C1 gene of maize, and wherein the activatable DNA construct comprises a synthetic promoter sequence comprising a CaMV 35S minimal promoter containing a TATA element fused at its 5' end to 10 concatemeric copies of the upstream activating sequence recognized by a GAL4 DNA binding domain.

11. The plant of claim 7, wherein the activatable DNA sequence is an AdSS antisense sequence.

INTERNATIONAL SEARCH REPORT

Internal application No
PCT/EP 98/07577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/52 C12Q1/68 C12N9/00 C12N15/62
G01N33/50 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 589 841 A (CIBA GEIGY AG) 30 March 1994 abstract, page 4, line 33; page 5, line 31; page 7, line 47-58; examples 14,15; claims	1,3,7,8 2,4,9,10
X Y	WO 97 30164 A (MEDICAL RES COUNCIL ;HASELOFF JAMES PHILLIP (GB); HODGE SARAH (GB)) 21 August 1997 pages 2-5; page 5, second paragraph; page 6,15; claims	1,3,8 2,4
X	WO 96 19576 A (CIBA GEIGY AG) 27 June 1996 claim 18; page 6,9	6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1999	03/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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P,X	EP 0 823 480 A (MAX PLANCK GESELLSCHAFT) 11 February 1998 see the whole document	1,3
E	WO 98 59062 A (DU PONT ;LIU ZHAN BIN (US); ODELL JOAN TELLEFSEN (US)) 30 December 1998 pages 13, line 23-35; page 18; examples ; fig. 3; claims	1

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/ 07577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: Claims 1-5,7-11

   Method of specifically activating a synthetic promoter and inducing the expression of an antisense ADSS sequence in an FI plant by crossing a first plant that is stably transformed with hybrid transcription factor with a second plant that contains the ADSS antisense sequence and a synthetic promoter that is activatable by said hybrid transcription factor.

2. Claim : Claim 6
   A method for the identification of ADSS herbicidal inhibitors by reactin a plant ADSS and ADSS substrate together with a potential inhibitor and determining the effect on ADSS enzymatic activity by comparing the rate of the enzymatic reaction in the presence and absence of the potential inhibitor.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible] .pplication No
PCT/EP 98/07577

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